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Title: Potential of a Fungus, *Acremonium sp.*, to Decolorize Pulp Mill Effluent

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This project explored the feasibility of using fungi in a constructed wetland for the treatment of pulp mill effluent. The effluent is high in dissolved lignins (some of which are chlorinated), which have proven very difficult to degrade biologically. Mindful of work done with the (terrestrial) white rot fungi, especially *Phanerochaete chrysosporium*, the question is asked, Is there a fungus which can tolerate submerged conditions while degrading a significant amount of dissolved lignins? Two fungal species with lignin-degrading capability were isolated from submerged films in a log pond. These fungi have been evaluated for decolorization potential under different environmental conditions.

Results of laboratory experiments show that one of these fungi, identified as *Acremonium sp.*, was capable of 44% decolorization of pulp mill effluent under sterile, submerged, room temperature conditions. The fungal decolorization was evaluated both in floating cultures and as a film inoculated on wood chips. In addition, bench-scale examination of the potential of this fungus to decolorize pulp mill effluent in non-sterile conditions was completed.

Potential of a Fungus, *Acremonium sp.*, to Decolorize Pulp Mill Effluent

by

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INTRODUCTION AND LITERATURE REVIEW

Lignin

Lignins are the structural molecules which give wood its resistance to microbial attack. There is no one molecule which can be identified as "lignin." Rather, the term includes a whole family of "complex macromolecule[s] originating from the random oxidative polymerization of hydroxylated cinnamyl alcohols" (Janshekar and Fiechter, 1983).

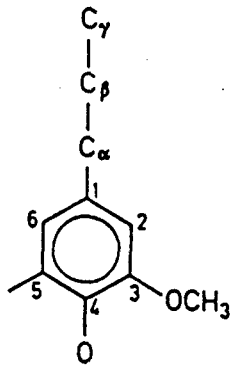


Figure 1. The lignin monomer. (From Janshekar and Fiechter, 1983, p. 129)

These molecules are some of nature's most recalcitrant to biodegradation, serving as "an important mechanism of disease resistance in plants" (Crawford, 1981). Hence the long life of downed wood on the forest floor, and its usefulness as a building material. We have created a wood products industry which exploits in many creative ways the unique properties of this complex organic molecule which stores an estimated 40% of the solar energy contained in plants (Janshekar and Fiechter, 1983).

In the industrial pulping process, mills extract wood fibers by exploding chips using combinations of chemical exposure and high pressure. The fibers, cleaned and bleached, are the pulp from which paper products are manufactured. The waste products from the fiber extraction and bleaching include many fragments of lignins, lignocelluloses, celluloses, and other wood components which have been removed from the fibers. These materials are released into the mill's wastewater stream as a kind of random, dark brown "soup" of byproducts, which can vary from batch to batch depending upon the wood species used, and pulping conditions (Biermann 1993). When pulp bleaching is performed with chlorine (the most common pulp bleaching agent in the U.S.), some of the organic compounds in the discharge are chlorinated and toxic.

Virtually all pulp mills in the U.S. have primary and secondary wastewater treatment systems which can effectively remove particulate matter and the readily degradable dissolved matter from this wastewater before discharging it. However, lignins are resistant to biodegradation and are not substantially altered in these treatment processes. "Bacterial aerobic and anaerobic treatment systems . . . cannot remove the dark color of kraft bleachery E_1 caustic extraction stage effluents (E_1 effluent) or degrade the high molecular weight chlorolignins they contain" (Archibald et al. 1990). The dark colored effluent is feared to have detrimental effects on receiving waters due to shading of algal communities (Howard et al. 1979), and may contribute to oxygen depletion with the often high BOD and especially high COD of the chromophores. Secondly, chlorinated lignins have been demonstrated to have acute and chronic toxicity (Kringstad and Lindström 1982, Walden et al. 1986), and to contain both mutagenic and carcinogenic compounds (Bjørseth et al. 1979, Lee et al. 1981, Walden et al. 1986).

Efforts to reduce the impacts of pulp mill effluent (PME) have included modifications of pulping process (especially to reduce or eliminate the use of chlorine in bleaching), chemical treatment of effluent, and further biological treatment. Improvement of industrial processes to generate lower pollutant concentrations and lower toxicity of the effluent is ongoing, and bears much promise for reduction of pollution by pulp mills. Some mills treat effluent chemically, then come under attack from the public about the water quality problems associated with high levels of chlorinated organics from the chemical treatment (Roger Sherwood 1993, Environmental Engineer, Pope & Talbot, Inc., personal communication).

Biological treatments of effluent have received much research attention (current reviews in Kirk and Chang 1990 and Leatham 1992). Early approaches examined bacterial degradation, in an effort to find the proper conditions or bacterial species with which recalcitrant constituents in pulp mill effluent could be broken down. While bacterial degradation of lignin can occur, it requires too much time to be considered feasible (Zimmerman 1990), on the order of 300 days under anaerobic conditions (Benner et al. 1984). Research on biological treatment of effluent has therefore centered on fungi. Lignin is one of the toughest natural polymers, but certain fungi have evolved the ability to decompose a variety of complex polymers, including lignin.

White-rot fungi

Most research on fungal degradation has centered on those fungi capable of producing "white-rot." These fungi can attack unaltered lignin polymers, cleaving C_α-C_β, ß-aryl ether, C_1-C_α, and aromatic rings (reviews in Higuchi 1989 and Schoemaker and Leisola 1990, newer developments in Valli and Gold 1991, Wariishi et al. 1991). Researchers have sought to employ these abilities in biopulping and biobleaching of wood, and in the

treatment of hazardous chemicals and wastes (summarized in Leatham 1992). When cultured in contact with pulp mill effluent under optimum conditions, white-rot fungi can decolorize by eliminating chromophores in the polymer and by degrading polymers down to lower molecular weight, colorless products which are soluble or even volatile. "The degradation shows no preference for molecular size and results in partial dechlorination of the polymer" (Sundman et al. 1981). White-rot fungi have been shown to decompose synthetic lignins to CO_2 (Kirk et al. 1978). Most research work has focused on one white-rot species, *Phanerochaete chrysosporium*, which is well suited to lab work due to its rapid growth and sporulation in culture. Another white-rot fungus which has shown promise for decolorization of PME is *Trametes (= Coriolus) versicolor* (Archibald et al. 1990, Bergbauer et al. 1990).

To date, none of the work done with *P. chrysosporium* for the decolorization of PME has been commercially applied. While a lag time between bench scale innovation and large scale application is not unusual, in this case the laboratory method may be impractical owing to the difficulty and high expense of keeping this terrestrial fungus alive in an aquatic environment. *Phanerochaete chrysosporium* is grown on Rotating Biological Contactors (RBCs) partially filled with wastewater. These units require energy as they continuously rotate plates covered with an established fungal mat. Rotation aerates the fungus, which is unable to decolorize unless periodically exposed to oxygen. Alternatively, the fungus can be grown as a fixed thin film on porous supporting materials in a trickling filter-type system. Both types of reactors are maintained at 39-40°C, and the effluent to be decolorized is adjusted to a pH of 4.5 (Joyce et al. 1984, Kirk et al. 1978, Leisola et al. 1984, Messner et al. 1990, Pellinen et al. 1988, Prouty 1990, Jaklin-Farcher et al. 1992). In practical terms, even the smallest pulp mills generate millions of gallons of effluent each day. The prospect of running so

much PME daily through this type of apparatus may be prohibitively expensive. Some researchers have suggested ultrafiltration as a precursor step, to simultaneously increase the concentration of effluent (and thereby increase the efficiency of color removal) treated in fixed-film reactors and reduce the volume run through them (Ek and Kolar 1990, Yin et al. 1990). Other researchers have examined the possibility of speeding up the fungal degradation with an ozone pretreatment step (Roy-Arcand et al. 1991), but these techniques carry their own significant costs.

Practical biological decolorization

Alternative systems for decolorizing PME in a safe, cost-effective manner are needed. Constructed wetland systems provide the possibility of an entirely different kind of fungal treatment. Constructed wetlands are a very promising technology for the low-cost, low-maintenance tertiary treatment of wastewater (US EPA 1987, US EPA 1988, Hammer 1989, Cooper and Findlater 1990, Olson and Marshall 1992, Water Board 1992, Lesley et al., In press). Applications to date have included municipal and industrial (acid mine drainage, sugar beet processing, etc.) wastewaters, and non-point source runoff (urban stormwater and agricultural). Suggested uses are expanding quickly as this technology evolves and matures. The pulp and paper industry is one proposed use which is being investigated in several sites worldwide (Allender 1984, Thut 1989, Knight 1993).

Constructed wetlands

Oregon State University's Department of Bioresource Engineering has a project with a local pulp mill, Pope & Talbot, Inc., to conduct research on, monitor, and evaluate the mill's pilot-scale constructed wetland facility.

Treatment of pulp mill effluent in a constructed wetland offers a unique opportunity for degradation of the dissolved lignins. It might be

possible to take advantage of the residence time within the wetland for color removal, if fungi capable of survival and effluent decolorization in wetland conditions can be identified. One attempt to use *P. chrysosporium* in constructed wetlands was unsuccessful (Hammer, In press). The work reported in this paper explores the possibility of using a fungus which is adapted to a submerged environment.

Submersible fungi

It is known that aquatic hyphomycetes (Fisher et al. 1983, Barlocher and Kendrick 1981) and other imperfect fungi (Prasad and Joyce 1991) can degrade lignin (Zare-Maivan and Shearer 1988, Jones 1976). These species are categorized as "soft-rot" fungi, and generally degrade cellulose as well as lignin. Biochemical characterization of enzymes and breakdown products produced by these organisms indicates that the mechanism of lignin degradation is different for soft-rotters than for white-rotters (Zare-Maivan and Shearer 1988, Prasad and Joyce 1991), though soft-rot fungi have been shown to be capable of degrading lignin model compounds to CO_2 (Haider and Trojanowski 1975). It has also been demonstrated that some basidiomycetes can decompose lignin under aquatic conditions (Jones 1982). Collection of biofilms from submerged wood in this investigation was intended to select for organisms which were adapted to the aquatic environment.

OBJECTIVES

A. Isolate lignin-degrading fungi from submerged wood in local natural areas.

B. Assess the ability of these fungi to decolorize pulp mill effluent in submerged, minimally engineered conditions.

MATERIALS AND METHODS

Collection of fungi

Biofilm samples were scraped from the surfaces of submerged wood in two creeks (relatively aerobic, flowing environment, Oak Creek and one of its unnamed ephemeral tributaries) and one local log pond (relatively anaerobic environment with no discernable flow, near the Mary's River in Philomath, OR). Samples were blended with a Waring Commercial Blendor (Model #33BL79, Dynamics Corp. of America, New Hartford, CT; five 5-second pulses) and diluted in distilled water (dH_2O) to 1:10, 1:100 and 1:1000, then combined with molten (45-50°C), sterile 1.5% malt agar (see Culture maintenance below) which was poured into Petri plates, where they solidified as the agar cooled. These sample plates were observed daily for growth of fungal colonies, which were removed and subcultured on fresh, sterile malt agar plates as they became visible. Sequential subculturing was repeated until apparently pure (no visible contamination) fungal colonies were obtained.

Isolation of potential lignin-degraders

In order to determine which of the collected fungi held potential for lignin degradation, two chemical dye decolorization screening tests were used. Dyes used for this purpose were synthetic molecules which are structurally similar to lignin. They were added to molten sterile agar, staining it a characteristic color. Most fungal colonies did not affect the color, because the dye structure is too complex to be affected by the degradative enzymes of most fungi. Lignin-degrading fungi, however, are usually identifiable at a glance, by a decolorized ring around the colony. The ring indicates that the fungus can degrade dye molecules and therefore may be capable of lignin breakdown (Glenn and Gold 1983).

Remazol Brilliant Blue (Sigma Chemical Co.) and Poly-R 478 (Sigma Chemical Co.) were filter sterilized (Millipore filter, 0.22 μm, Millipore Corp., Bedford, MA) and added aseptically to a final concentration of 0.02% (w/v) to liquid malt agar that had been autoclaved and cooled to 45-50°C in a New Brunswick AgarMatic Bench-Top agar sterilizer (New Brunswick Scientific, Edison, NJ) (Freitag and Morrell 1992). Upon cooling, these solidified into brightly colored (Remazol Brilliant Blue yielded blue, Poly-R 478 yielded pink) dye plates. Fungi were aseptically subcultured onto these plates, maintained at 20-23°C, and their growth observed over a five-week period for qualitative signs of decolorization.

Culture maintenance

Most fungal cultures were maintained on malt extract agar, and were subcultured monthly onto fresh medium. Plastic Petri plates (90 mm in diameter) were filled with 20 ($\pm$ 1) ml of an autoclaved (New Brunswick AgarMatic) medium containing 15 g malt extract (Difco Laboratories, Detroit, MI) and 10 g agar (Sigma Chemical Co., St. Louis, MO) per liter. Selected fungi were maintained on Difco Bacto Potato Dextrose Agar (Difco Laboratories, Detroit, MI) (39 g/l). All plates were poured with a New Brunswick PourMatic MP-320 plate pouring system.

Despite periodic subculturing, cultures twice developed mite contamination, which was successfully removed once with p-dichlorobenzene fumigation followed by subculturing once per week for three weeks. In the second mite invasion, mites did not respond to p-dichlorobenzene, so physical purification of the culture was achieved by frequent (twice per week) subculture by removing colonies under a dissecting microscope (to see the very small colony and to avoid picking up mites and eggs) over a three week period.

Liquid cultures were used for growing fungal biomass for use in the wood chip assays. Six plugs (8 mm diameter) of fungus-colonized agar and 50 ml of 1.5% sterile malt extract (Difco Laboratories) solution were aseptically added to 250-ml Erlenmeyer flasks. The flasks were capped with a sterile cotton plug and sterile aluminum foil, then shaken continuously for 7-10 days at 80 rpm and 20-23°C to produce compact fungal masses for maceration.

Identification of *Acremonium sp.*

Initially, work was performed with two collected fungal species (dubbed "Angelou" and "Barbara") without regard for identification. When one species ("Barbara") proved able to decolorize PME by 42%, identification became desirable. Growth rate was measured on malt agar and potato dextrose agar plates. The dense, constricted, white to yellow appearance of the colonies on malt agar led to suspicions of bacterial contamination, so cultures were grown on streptomycin-laced agar plates (1 g Agri-strep per 500 ml standard malt agar solution).

The fungus was also cultured on gallic and tannic acid agar plates. These plates provide a diagnostic medium, helping to identify species by presence or lack of fungal growth and/or discoloration. Gallic or tannic acid agar was prepared by adding 5 g of gallic acid crystals (Mallinckrodt Chemical Works, St. Louis, MO) or tannic acid reagent powder (Matheson Coleman & Bell, Norwood, OH) to 150 ml sterile distilled water, mixing thoroughly. Malt agar solution (850 ml distilled water, with 20 g agar and 15 g malt extract) was autoclaved, then cooled to 45-50°C and combined with the acid solution. The resulting mixture was stirred well and hand-poured into sterile plastic Petri plates while still molten.

The presence of laccase, an important enzyme system typical of many white-rot fungi was evaluated by adding a few drops of 0.1 M α-naphthol (in 95% ethanol) directly to the growing edge of 5 to 7 day old fungal colonies on 1.5% malt agar plates grown at 20-23°C.

In addition, the fungus was subjected to microscopic examination of mycelial squash mounts from malt agar culture. It was then grown on slide cultures and the slides were examined for identifiable conidiogenous structures. These results were compared with available keys (Gams 1971, Ainsworth et al. 1973, Wang and Zabel 1990).

Experimental design

Decolorization assays to determine the ability of fungi to decolorize PME were conducted in six sets. Four sets of nutrient assays were done, in December 1991 (Run 1), March 1992 (Run 2), June 1992 (Run 3), and December 1992 (Run 4). The first three runs tested several carbon and nitrogen sources and levels (Table 1). Run 4 repeated two previously tested nutrient conditions, while testing the effects of aeration and non-sterile incubation on decolorization. In addition, two sets of chip assays were performed, in July 1992 and August 1992, to examine the feasibility of fungal colonization of wood chips as an immobilizing medium for PME decolorization (Table 2 summarizes chip assay treatments).

Each set was executed with a fresh batch of PME collected from Pope & Talbot's secondarily treated effluent no more than two days before the start of the experiment. Effluent was stored in a sealed container at 5°C until use.

Table 1. Nutrient assay conditions.

Nutrient Code	Nutrients added	Fungi tested	In Run #
STERILE, NON-AERATED			
a	5 g/l fructose 50.4 mg/l urea	A, B, C, D, Control*	1
b	100 g/l fructose 100 mg/l urea	A, B, C, D, Control	1
c	20 g/l fructose 50.4 mg/l urea	A, B, C, D, Control	1
d	no fructose 50.4 mg/l urea	A, B, C, D, Control	1
e	no fructose no urea	A, B, C, D, Control	1 & 2
f	10 g/l fructose 200 mg/l urea	A, B, C, D, Control	2
g	10 g/l glucose 200 mg/l urea	A, B, C, D, Control	2, 3, & 4
h	20 g/l fructose 200 mg/l urea	A, B, C, D, Control	2
i	5 g/l glucose 200 mg/l urea	A, B, C, Control	3
j	10 g/l glucose 100 mg/l urea	A, B, C, Control	3 & 4
k	200 g/l glucose 200 mg/l urea	A, B, C, Control	3
STERILE, NON-AERATED, SAMPLED OVER TIME			
g	10 g/l glucose 200 mg/l urea	A, B, C, Control	3
STERILE, AERATED			
g	10 g/l glucose 200 mg/l urea	B	4
j	10 g/l glucose 100 mg/l urea	B, C, Control	4
NON-STERILE, AERATED & NON-AERATED			
g	10 g/l glucose 200 mg/l urea	B	4
j	10 g/l glucose 100 mg/l urea	B, C, Control	4

* A=Angelou, B=*Acremonium sp.* , C=*Phanerochaete chrysosporium*, D=*Phlebia subserialis*, Control=no fungus added

Measurement of PME color

Color was measured using a previously described procedure (NCASI, 1971) as absorbance at 465 nm (Abs_{465}), using a Milton Roy Spectronic 301 spectrophotometer. Samples were filtered in several stages to pass a 0.8 μm membrane (all sub-μ filters Millipore), and then adjusted to pH 7.6. For initial color of sterile effluent, samples were filtered to 0.2 μm before measurement. The results were calibrated using a platinum-cobalt color units (PCU) standard (Color Standard Solution, 500 Platinum Cobalt Units, Hach Company, Loveland, CO), with the experimentally-derived linear relationship: $(4174.459 \times Abs_{465}) - 18.401 = PCU$.

Sterile nutrient assays

Four day to 11 day old fungal plate cultures were used for all nutrient assays. PME was filtered in several stages to pass a 0.45 μm filter, then micronutrients and carbon and nitrogen sources (nutrients) were added. The micronutrient solution contained the following components (per liter PME, adapted from Michel et al. 1991): nitriloacetate, 0.14 g; NaCl, 0.07 g; $FeSO_4 \cdot 7H_2O$, 0.007 g; $CoCl_2 \cdot 6H_2O$, 0.013 g; $ZnSO_4$, 0.07 g; $CuSO_4 \cdot 5H_2O$, 0.0011 g; $AlK(SO_4)_2 \cdot 12H_2O$, 0.0007 g; H_3BO_4, 0.0007 g; $Na_2MoO_4 \cdot 2H_2O$, 0.0007 g; $MnSO_4$, 0.012 g; thiamine HCl, 0.001 g. Addition of micronutrients may not have been necessary, given the presence of trace minerals leached from the pulped wood (Pope & Talbot effluent analyses, unpublished, Halsey, OR), but they were added to <u>all</u> tests, to eliminate the possibility of micronutrient limitation. The presence of manganese and thiamine are considered especially important for lignin-degrading organisms (Kirk et al. 1978, Bonnarme and Jeffries 1990, Brown et al. 1990, Brown et al. 1991, Michel et al. 1991).

Runs 1 and 2 tested four fungal species: Angelou, *Acremonium sp.*, *P. chrysosporium* (BKM F-1767), and *Phlebia subserialis* (SS3). Run 3 tested all

of these species except *P. subserialis*, and Run 4 tested *Acremonium sp.* and *P. chrysosporium*.

Several carbon and nitrogen sources and levels were examined (Table 1). The resulting solutions were filtered to 0.2 μm; 50 ml were aseptically measured into each sterile 250-ml Erlenmeyer flask, and capped with sterile cotton and foil. Four (Runs 1-3) or five (Run 4) replicates were produced for each treatment. Flasks (except controls) were each inoculated with 5 agar plugs (8 mm diameter) cut from actively growing edges of the test fungus. They were incubated under stationary conditions, at room temperature (20-23°C) for 14 days. Runs 1-3 were incubated on open counter space in the laboratory near an east-facing window, but concerns about fluctuating laboratory temperatures in December 1992 caused Run 4 flasks to be incubated in a closed incubator. The supernatant was then filtered to 0.8 μm, the pH adjusted to 7.6, and Abs_{465} of the resulting solution was measured and recorded.

Decolorization rate

One extra set of sterile nutrient assays at nutrient level g (in Table 3, distinguished as "g2") was performed in Run 3, to examine rate of decolorization over the 14-day decolorization period employed in other sets. Extra replicate flasks (a total of 14) were set up for each treatment; two replicates of each treatment were terminated at the 1, 3, 5, 7, and 10 day points, leaving four replicates to be analyzed at the 14 day point. The flasks used for this treatment were "mini" versions of the sterile nutrient assay flasks already described: sterile 125-ml Erlenmeyer flasks contained 25 ml of nutrient-added, 0.2 μm-filtered PME, inoculated with 2.5 fungal agar plugs (8 mm diameter). These flasks were prepared and incubated identically to those described in the preceding section. At the indicated termination points, two flasks were randomly selected from each treatment, the

supernatant filtered, adjusted, and analyzed as described in the preceding section.

Non-sterile nutrient assays

The use of sterile PME, while useful for controlling variables, does not accurately reflect the conditions in commercial operations. Eight non-sterile treatments were executed as part of Run 4. Four treatments of aerated and four treatments of non-aerated flasks were prepared, comprised of 5 replicates of *Acremonium sp.* at two different nutrient levels (g and j), 5 replicates of *P. chrysosporium* at one nutrient level (j), and 3 replicate control flasks (see Table 1). Procedures for non-sterile assays were identical to those used for the sterile nutrient assays, except that PME was not filtered before inoculation. All glassware was sterile, and division of PME into 250-ml flasks was performed aseptically, to prevent introduction of fungi or bacteria of laboratory origin into flasks.

Aerated nutrient assays

Aeration was introduced into eight sets of flasks in Run 4 to see if decolorization rates could be enhanced. Four treatments of sterile and four treatments of non-sterile aerated flasks were prepared, comprised of 5 replicates of *Acremonium sp.* at two different nutrient levels (g and j), 5 replicates of *P. chrysosporium* at one nutrient level (j), and 3 replicate control flasks. Aerated flasks were prepared identically to sterile or non-sterile nutrient assays described in preceding sections, but they were stoppered with rubber stoppers using a modification of a previously described method (Kirk et al. 1978). The stopper gas outflow structure was modified because it was not necessary to quantify or analyze outflow; outflow tubes fitted with sterile Pasteur pipettes each containing a sterile cotton plug, to prevent entry of organisms through the outflow tube were instead employed. Aerated flasks

were flushed for 15 minutes every 3 - 4 days with 100 - 200 ml per minute of pure O_2.

Chip assays

Douglas-fir chips from Pope & Talbot (mechanically chipped into random shapes, 4.7 x 1.5 x 0.4 cm average size) were oven-dried for 24 hours at 100°C, then vacuum-soaked for 30 minutes at 686 mm Hg in distilled water with micronutrients (see micronutrient solution previously described), 1.5% malt with micronutrients, or 1% glucose/0.01% urea nutrient solution with micronutrients (see ensuing description, and Table 2 for listing of replicates). Sixty g of soaked chips were drained, then placed in clean, dry 1-liter Erlenmeyer flasks. Flasks were plugged with cotton plugs and capped with aluminum foil prior to sterilization (121°C for 25 minutes).

The chips were used in two assays. Chip assay 1: Plugs of *Acremonium sp.* from 3 flasks of shaken liquid culture (previously described in Culture maintenance) were aseptically macerated with 200 ml sterile dH_2O in a Waring Commercial Blendor (five 5-second pulses). Thirty ml of solution were diluted with 100 ml sterile dH_2O, and added to each of three replicate chip flasks containing chips soaked in 1.5% malt, or 1% glucose/0.01% urea solution (total liquid volume to barely cover chips). Sterile dH_2O (no fungus) was added to each of 3 chip flasks to serve as controls. All flasks were manually swirled for 3 minutes, to completely mix chips with fungi and water. Flasks were incubated in stationary conditions for 8 days at room temperature (20-23°C), then the liquid was aseptically decanted.

Chip assay 2: *Acremonium sp.* from 14 liquid culture flasks was macerated with 3.5 l dH_2O, and 125 ml of this fungus suspension was added to each of 27 chip flasks. Fungi from 6 *P. chrysosporium* liquid culture flasks were blended with 2.0 l dH_2O, and 125 ml of this suspension were added to

each of 15 flasks. These flasks were incubated from 2 - 12 days at room temperature (20-23°C), then the liquid was aseptically decanted. Table 2 delineates the assignment of treatments and replicates.

In the decolorization phase, nutrients (1% glucose/0.01% urea, corresponds to nutrient level j) and micronutrients (same composition as micronutrients used in sterile nutrient assays) were added to 30 l PME. Twenty-two l of this solution was then filtered in stages to pass a 0.2 μm filter. Five hundred ml were aseptically added to each of 43 sterile chip flasks (five replicate fungus flasks, eight control replicates, see Table 2). Eight l of the nutrient-supplemented PME was left non-sterile; 500 ml were aseptically added to each of 14 non-sterile chip flasks (seven *Acremonium sp.* replicates, seven control). Color was measured after 14 days of stationary, room temperature incubation, as previously described.

Table 2. Chip assay treatments.

Chip assay #	Incubation time of fungus on chips	Chip soaking solution	Sterile/ non-sterile	Fungus	# Replicates
1	--	dH_2O	Sterile	Control	3
1	8 days	1.5% malt	Sterile	*Acremonium sp.*	3
1	8 days	1% glucose, 0.01% urea	Sterile	*Acremonium sp.*	3
				Total # of flasks in assay =	9
2	--	1.5% malt	Sterile	Control	8
2	2 days	1.5% malt	Sterile	*Acremonium sp.*	5
2	4 days	1.5% malt	Sterile	*Acremonium sp.*	5
2	9 days	1.5% malt	Sterile	*Acremonium sp.*	5
2	12 days	1.5% malt	Sterile	*Acremonium sp.*	5
2	2 days	1.5% malt	Sterile	*P. chrysosporium*	5
2	4 days	1.5% malt	Sterile	*P. chrysosporium*	5
2	9 days	1.5% malt	Sterile	*P. chrysosporium*	5
2	--	1.5% malt	Non-sterile	Control	7
2	9 days	1.5% malt	Non-sterile	*Acremonium sp.*	7
				Total # of flasks in assay =	57

RESULTS AND ANALYSIS

Isolation of potential lignin-degraders

Two morphologically distinct fungi were indicated as possible lignin-degraders, by positive tests (the presence of decolorized rings around colonies) on both indicator dyes. None of the other (over 200) fungal colonies obtained from the field gave a positive dye test on either dye. Both of the decolorizers (temporarily labeled "Angelou" and "Barbara"; Barbara was later identified, see below) were from log pond samples.

Identification of *Acremonium sp.*

Growth rate of fungal colonies on malt agar was quite slow, 16.5 mm diameter in 10 days and a maximum colony diameter of 19.5 mm. On potato dextrose agar, colonies grew to 17 mm diameter in 10 days and a maximum colony diameter of 39 mm. Colonies did not expand beyond these maximum colony dimensions. Growth on gallic and tannic acid agar was much slower than growth rate on malt agar, and showed no discoloration reaction. Laccase test was also negative.

The isolate would not grow in streptomycin medium. Streaking of mycelium on malt agar plates, growth in liquid cultures and microscopic examination throughout the 18-month experimentation period showed no evidence of bacterial contamination.

Microscopic examination showed no clamp connections; the fungus was found to be an imperfect phialidic species. Slide culture examination showed phialides and conidia (Figure 2), which were noted and compared with descriptions available in taxonomic keys (Ainsworth et al. 1973, Wang and Zabel 1990). Drawings of structures were then compared with Gams

(1971); the best fit was with *Acremonium alternatum* (tentative identification, requiring confirmation).

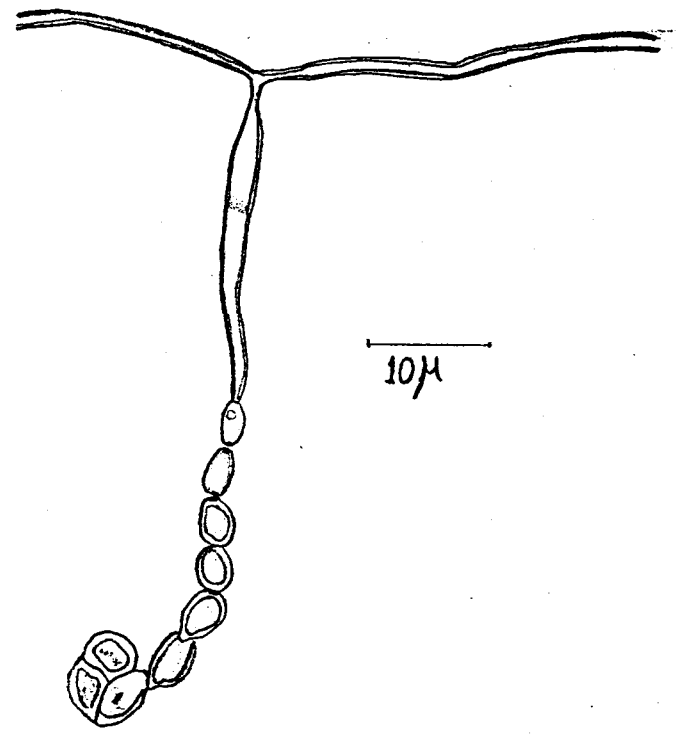


Figure 2. Drawing of phialidic structure from *Acremonium sp.* isolate used in this experiment.

Nutrient assays: individual runs

Appendix B shows all of the raw data from individual Runs 1-4, reported in PCU. Also in Appendix B, all treatments in each run are compared to the control mean using a Bayes Least Significant Difference (Bayes LSD) separation of means (Petersen 1985). This test allows separation of statistically significant treatments, which are reported in Table 3. Table 3 lists the significant treatments, and reports the percent

decolorization obtained (± 95% confidence interval), calculated as compared to initial effluent color.

December 1991 - Run 1: Three fungi, Angelou, *Acremonium sp.*, and *Phanerochaete chrysosporium* decolorized PME significantly more than the control (no fungus) under two nutrient regimes (b and c). Decolorization ranged from 10-15% (Table 3), while control (no fungus) decolorized PME by 6.1%.

March 1992 - Run 2: Substitution of glucose for fructose as a carbon source (nutrient level g) increased PME decolorization by *Acremonium sp.* to 41.89%. Results from other fungi at all tested nutrient levels and *Acremonium sp.* at other tested nutrient levels did not differ statistically from the control (no fungus), which reduced color by 4.5%. Forty-two percent decolorization after 14 days represents a visible difference in color (Figure 3), indicating that fructose might be an undesirable carbon source.

Figure 3. Example of PME decolorization in Run 2 after 14 days of incubation. From left to right: control, *Acremonium sp.*, and *P. chrysosporium*.

Consequently, glucose was used as the added carbon source in all subsequent trials.

June 1992 - Run 3: This trial evaluated the effects of four different nutrient regimes (g, i, j, and k) on decolorization. Nutrient level g was performed twice (two full sets of replicates, g1 and g2), one of these sets (g2) contained extra replicates to permit evaluation of decolorization rate over the 14-day incubation period. No significant decolorization was noted with Angelou at nutrient level g. Most decolorization by *Acremonium sp.* occurred in the first five days (Figure 4). When fitted to an exponential decay model ($C = C_o e^{-kt}$) using non-linear regression, the best-fit rate constant for decolorization by *Acremonium sp.* was determined to be 0.0438 (Figure 5). Decolorization by *P. chrysosporium* occurred only after an initial three-day lag (Figure 4), which agrees with the growth phase reported in the literature (Keyser et al. 1978, Kirk et al. 1978, Eaton et al. 1980, Yin et al. 1989). Using the 3-day point as initial color value, the *P. chrysosporium* decolorization rate data was fitted to the exponential decay model with non-linear regression and the best-fit rate constant for decolorization was determined to be 0.0491 (Figure 6).

Table 3 summarizes the significant final 14-day decolorization results. At all nutrient levels tested, *Acremonium sp.* decolorized PME by 29-44%. However, differences between trials at the same nutrient conditions were noted. For example, nutrient condition g resulted in 28.86 and 37.73% decolorization (statistically significant difference) by *Acremonium sp.* in two separate trials, suggesting that variables other than carbon source may significantly alter decolorization. *Phanerochaete chrysosporium* significantly decolorized the set at nutrient level g which was examined at intermediate time points (g2), in which it decolorized 28.52% by the end of the 14-day decolorization period, but significant decolorization was not observed in the

Figure 4. Decolorization rate.
Nutrient level g: 1% glucose/0.02% urea

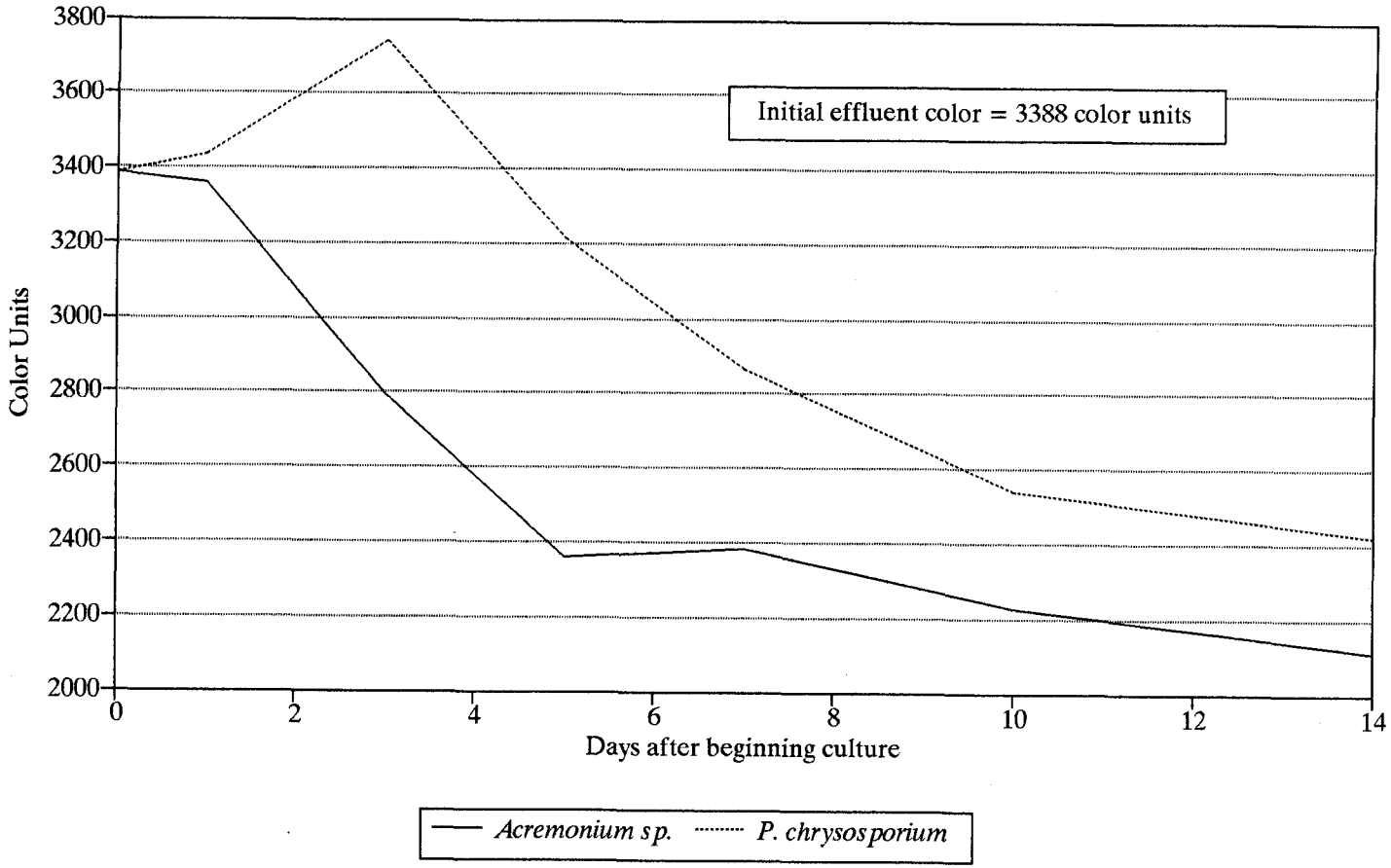


Figure 5. Exponential decay model:
Acremonium sp.

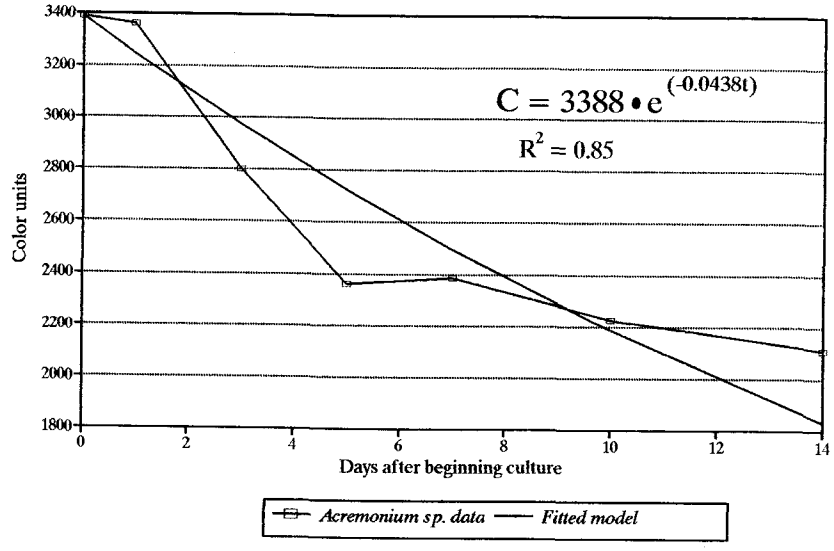
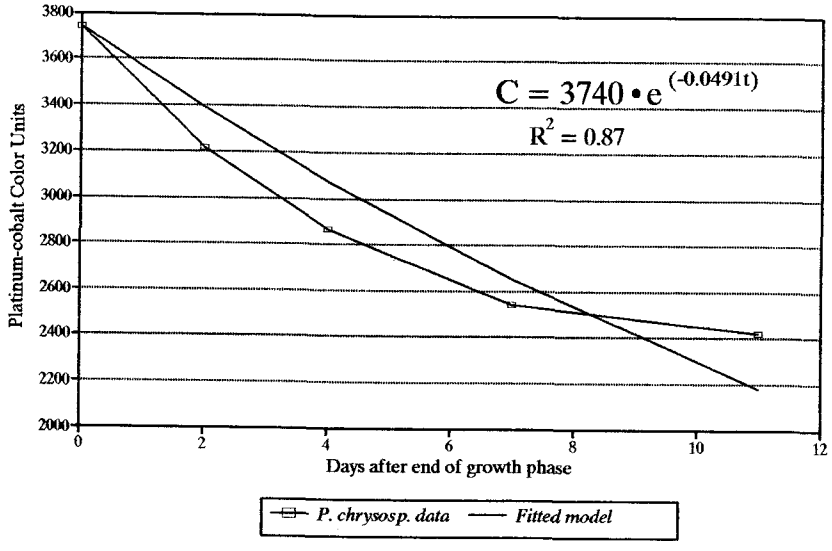


Figure 6. Exponential decay model:
P. chrysosporium



other g (g1) trial. *P. chrysosporium* also significantly decolorized at nutrient level j (35.89%). Angelou decolorized by 31.39% at nutrient level j, but not significantly at other nutrient levels. Control decolorization in this run was 14.37%.

December 1992 - Run 4: Statistical comparisons were made between treatment sets and controls of matching conditions, e.g., non-sterile, aerated fungal flasks were compared to non-sterile, aerated control flasks, etc. Only sterile *Acremonium sp.* showed significant color removal compared to controls, producing 19-46% color removal (see Table 3) while control color removal ranged from -2.1% (adding color) to 15.4%. Both nutrient levels tested showed similar results, with no statistically significant difference between them (Petersen 1985). A significant difference was observed between aerated and non-aerated *Acremonium sp.*, indicating that aeration significantly improved decolorization.

Table 3. Decolorization of pulp mill effluent by fungi under varying nutrient conditions. Summary of significant** nutrient assay results.

Run #	Fungus	Nutrient code(s)	% Decolorization§
1	Angelou	b	12.98 ± 4.17
		c	15.19 ± 3.99
	Acremonium sp.	b	12.03 ± 0.93
		c	11.97 ± 1.20
	P. chrysosporium	b	13.46 ± 3.77
		c	10.70 ± 7.07
2	*Acremonium sp.*	g	41.89 ± 2.04
3	Angelou	j	31.39 ± 26.85
	Acremonium sp.	g1	28.86 ± 14.05
		g2	37.73 ± 9.78
		i	43.22 ± 1.65
		j	44.36 ± 1.23
		k	42.76 ± 3.29
	P. chrysosporium	g2	28.52 ± 18.52
		j	35.89 ± 14.16
4	*Acremonium sp.*		
	Sterile, non-aerated:	g	23.06 ± 10.65
		j	19.23 ± 14.41
	Sterile, aerated:	g	42.78 ± 11.38
		j	46.10 ± 2.10

**Significantly different from matched controls, at 0.01 signif. level
§Compared to initial color, ± 95% confidence interval

Nutrient assays: selected contrasts

Selected data from Runs 3 & 4 were combined for the purpose of constructing contrasts which might yield more statistically significant information (Steel and Torrie 1960). Data were chosen as follows: Run 3, all *Acremonium sp.* and *P. chrysosporium* data; Run 4, all sterile data. Selected data were adjusted to matched controls, to remove the effluent batch effect, with the formula (Control-Data value)/Control. Of the ten contrasts analyzed from selected data from Runs 3 and 4, seven show statistically significant results (Table 4). Variability between runs, aeration, fungal species (*Acremonium sp.* and *P. chrysosporium)*, the interaction between fungal species and aeration, and the interaction between nutrient level and fungal species are factors which partly explain the significant differences seen among treatments.

Table 4. Summary of significant contrasts analyzed from nutrient assay data of pulp mill effluent decolorization by fungi.

Contrast #	Description	F-value	Signif. level
1 - 3	Variability between Run 3 & Run 4	8.84	0.01
4	Effect of aeration	20.4	0.01
5	Interaction of aeration & fungal species	4.29	0.05
6	Difference between *Acremonium sp.* and *P. chrysosporium*	99.0	0.01
8	Interaction of nutrient level and fungal species	12.2	0.01

Chip assay results

Chip assay 1 produced no statistically significant decolorization. Chip assay 2, however, showed significant decolorization. Under sterile conditions, both fungi (*Acremonium sp.* and *P. chrysosporium*) decolorized significantly more than controls at all incubation times tested (Table 5), except *Acremonium sp.* at a 4 day incubation (2 PCU shy of significance at 0.05 level). Decolorization ranged from 11-15%, showing no significant differences between the treatments, while average control decolorization was 1.81%. Exposure of the non-sterile effluent to *Acremonium sp.* for 9 days produced 28.20% decolorization, a significantly higher decolorization level than the non-sterile control, which decolorized by 19.99%.

Six contrasts were constructed to statistically apportion the significant treatment effects (Steel and Torrie 1960). The full list of contrasts tested appears in Appendix B. Three of these contrasts were found to be significant: 1) Non-sterile PME with *Acremonium sp.* added vs. non-sterile PME controls (0.05 significance level), 2) Sterile PME with fungus added vs. sterile PME controls (0.01 significance level), and 3) Non-sterile treatments and controls vs. sterile treatments and controls (0.01 significance level). While the first two were implicit in the preceding analysis of separation of means, the third adds the information that non-sterile conditions yielded significantly greater treatment than sterile conditions.

Most of the flasks in both chip assays (fungal and controls) showed contamination with *Penicillium sp.*, and/or *Aspergillis sp.* Since contamination was not visible in any of the nutrient assays (conditions were very similar, but no chips used), it was concluded that the initial sterilization of the wood chips was insufficient. Chips may somewhat protect resident fungal mycelium or spores from heat or may encourage formation of heat resistant resting structures.

Table 5. Pulp mill effluent decolorization after 14 days exposure to fungi incubated for varying times on douglas-fir chips. Summary of significant* results.

Fungus & Sterility	Incubation time (days)	% Decolorization§
Acremonium sp.		
STERILE	2	11.00 ± 6.44
	9	13.05 ± 8.70
	12	15.31 ± 10.33
NON-STERILE	8	28.20 ± 1.55
P. chrysosporium		
STERILE	2	12.07 ± 2.77
	4	12.05 ± 1.55
	9	13.27 ± 3.32

*Significantly different from matched controls, at 0.05 significance level
§Compared to initial color, ± 95% confidence interval

DISCUSSION

In these experiments, *Acremonium sp.* showed better color removal over a wider range of culture conditions than the other fungal species tested. Most encouraging from the standpoint of possible practical applications was the successful decolorization (best result: 44.36 $\pm$ 1.23% from initial color, compared with 14.37 $\pm$ 4.45% observed in control) achieved at room temperature, with no pre-adjustment of pH or aeration.

The ability of this *Acremonium* species to perform substantial decolorization of pulp mill effluent under simple, minimally engineered conditions is intriguing, but also daunting. Little is known about this genus, finding expertise to positively identify species is difficult, and it is even suggested that some of the described species should be re-assigned to other genera. Members of the genus have been found ubiquitously, as saprophytes in many habitats or as pathogens of plants and animals (Samuels 1976). Studies of aquatic habitats have described *Acremonium* and *Cephalosporium* (confusion and cross-categorization occur between these two genera) species in sewage and other polluted waters, on red mangrove seedlings and cooling tower timbers in marine environments, as well as lignicolous species in freshwater environments (Jones 1976).

Confirmation of tentative identification (*Acremonium alternatum*) is needed, as well as teleomorph classification, before the development of a field application for this fungus is attempted. Potentially useful fungal species should always be tested for vertebrate pathogenic activity. Rare, opportunistic fungal pathogens are becoming more of a health hazard as more people develop compromised immune systems, while the tests for determining pathogenicity are simple (William Denison, Assoc. Prof., Dept. of Botany & Plant Pathology, Oregon State U. 1993, personal

communication). Furthermore, organisms inhabiting receiving waters might also be vulnerable to opportunistic fungi. Before development of a field application, it will be necessary to examine effects on organisms such as fish, invertebrates, and benthic algae, to avoid disrupting the balance of natural systems which may already be stressed by the presence of wastewater discharge. Investigation of pathogenicity of this fungal species was beyond the scope of this study.

In addition to questions about fungal effects on receiving waters, there may be valid concerns about the effects of altered wastewater constituents. Biochemical analysis of breakdown products was not performed in this study, but it should be carried out at an early stage in the development of this biotechnology. *P. chrysosporium*, other white-rot fungi, and soft-rot fungi are capable of degrading lignin to carbon dioxide (Kirk et al. 1976, Haider and Trojanowski 1975) but if exposure time is insufficient, that process will be incomplete and lignin fragments will be released. The ecological and environmental impact of these fragments should be assessed; it is possible that the lignin fragments (especially if halogenated) might be more hazardous, or less labile than the original effluent material.

Recognizing that more investigation is needed, nonetheless the results of this study warrant scrutiny. Aeration of medium has previously been shown to enhance decolorization by *P. chrysosporium* (Kirk et al. 1978, Eaton et al. 1980, Eriksson and Kirk 1985, Reid et al. 1985, mechanism described in Dosoretz et al. 1990). These results support that conclusion; contrast analysis comparing aerated vs. non-aerated flasks show highly significant enhancement of decolorization under aeration.

P. chrysosporium (BKM F-1767) has been shown to have two distinct metabolic phases when cultured under optimum decolorizing conditions

(Keyser et al. 1978, Kirk et al. 1978, Eaton et al. 1980, Yin et al. 1989). The first phase is labeled "growth," lasting 2 - 4 days, during which little or no decolorization occurs. Then, in nitrogen-limited conditions (repeated in this study), "secondary metabolism" occurs (Bu'Lock 1975), during which decolorization takes place. Two weeks was chosen as an incubation time in this experiment in order to allow a growth phase to elapse. Analysis of decolorization rate (Figure 4) shows an initial lag for *P. chrysosporium*, which appears to correspond with the reported growth phase. But *Acremonium sp.* does not demonstrate a growth phase. Decolorization proceeds from the first day and levels off at the five-day mark. It should be emphasized that each decolorization rate data point in Figure 4 is the average of only two replicates, nevertheless these data suggest that the two species may have different metabolic responses to these culture conditions. New strains of *P. chrysosporium* are being screened which have shorter lag periods before decolorization, but it remains to be seen how successful these strains will be (Tien and Myer 1990, Boominathan and Reddy 1992). While a slightly higher decolorization rate was observed in *P. chrysosporium*, lack of a lag phase would favor the use of *Acremonium sp.* in decolorization applications.

It has been shown that a carbon source is necessary for *P. chrysosporium* to decolorize PME (Kirk et al. 1976, Eaton et al. 1980). Archibald et al. (1990) concluded that "inexpensive sugar refinery or brewery wastes are excellent growth and decolorizing substrates" for *Coriolus versicolor*, but no analysis of the sugars metabolized from these wastes was included. Results of the current experiments indicate that the source of carbon makes a crucial difference to the ability of three fungi - Angelou, *Acremonium sp.*, and *P. chrysosporium* - to decolorize PME. Fructose was significantly less effective than glucose in supporting decolorization by these fungi.

In the assays performed, fungal biomass was introduced as mycelium in agar plugs. The resulting fungal biomass was not quantified; it may not have been enough to promote optimal levels of decolorization. Perhaps most important for decolorization, the ratio of fungal surface area to treated liquid volume may not have been high enough for optimum treatment. Initially, 5 plugs of 8 mm diameter and 5 mm thickness provided a maximum (assuming complete fungal coverage of plug surface area) of 1131 mm^2 surface area per 50 ml PME volume. Over the 14 day decolorization period, growth of fungal mycelium was seen to increase plug surface area in some flasks to as much as 14 mm diameter and 8 mm thickness per plug, a total of 3299 mm^2 surface area per 50 ml PME volume, or only a three-fold increase. Growth of *Acremonium sp.* (but not the other fungal species) during the decolorization period was sometimes observed to occur not only on the surface of the plugs, but in many tiny (1 mm diameter) free-floating masses. While not specifically evaluated in these experiments, this growth pattern has the potential for creating a much higher increase in effective fungal surface area. Species capable of rapid sporulation and germination would have a major advantage in a field application. Further studies of optimum fungal surface area to treatment volume ratios would be beneficial for assessing the usefulness of this fungus.

Run 4 repeated culture conditions g and j (see Table 1), and although experimental values were significantly different from the control, there was much less decolorization in sterile fungal and control flasks than these conditions yielded in earlier Run 3. This run was executed in insulated dark chambers to protect samples from widely fluctuating laboratory temperatures occurring at that time (maintaining the flasks at 20-23°C). Ultraviolet light can decrease the color of PME (McKelvey and Dugal 1975, Roy-Arcand and Archibald 1993), and the absence of light in Run 4 may help to explain the differences noted in the controls.

Differences between PME collected from the Pope & Talbot secondary treatment lagoon outflow in summer compared to winter provide an alternative explanation of the lower color removal in winter PME. Run 4 used PME collected in December 1992, while Run 3 used PME collected in June 1992. Pulp mill effluent composition can vary significantly between spring and summer, due to seasonal variations in secondary treatment conditions and mechanisms (Bryant and Amy 1989, Pope & Talbot unpublished data 1992-1993). A greater fraction of high-molecular-weight compounds is expected in the winter effluent due to lower bacterial activity in secondary treatment lagoons. This "tougher" effluent should be more resistant to degradation; an effect which could partially explain variability between Run 4 and Run 3 decolorization.

Anomalous differences between the g1 and g2 sets in Run 3 are difficult to explain. Separation of means with Bayes LSD (Petersen 1985) shows significant differences between g1 and g2 in both *Acremonium sp.* and *P. chrysosporium* treatments. No substantial difference in conditions of the two sets was intended. As reported in Materials and Methods, the g2 flasks were one-half the size of g1 flasks, but contained also one-half the PME volume and one-half the fungal biomass introduced, resulting in approximately the same fungal surface area to PME volume ratio. The method of inoculating flasks with colonized fungal agar plugs may have caused these differences. Plugs cut from the growing edges of colonies are not uniform: variability in the amount of fungal biomass introduced in this way and/or the variability in metabolic state of the introduced mycelium may contribute to the observed differences. This theory also suggests another explanation for inter-run differences discussed in the preceding two paragraphs. Additional studies of the conditions used in these experiments might profitably test other ways of introducing fungal biomass into PME for

treatment (e.g., inoculation with spore suspensions), to isolate that source of treatment variability.

In a flowing wastewater treatment situation, fungal treatment will require some type of immobilization, so that fungal biomass does not simply flow out of the reactor with the wastewater. Chip assays were performed in an attempt to identify a practical application of *Acremonium sp.* to PME decolorization. Results of the sterile chip assays were compromised by contaminating fungi, suggesting a requirement of sterile inoculation of whatever medium is used in an application. However, the presence of test fungi significantly enhanced decolorization of PME over the control flasks (which were also contaminated). In this first attempt to immobilize this fungus, ready availability of Douglas-fir chips made the most practical choice of medium. Future work might explore differing immobilization media, to determine if there is a better surface on which to grow *Acremonium* biofilms.

Fungal enhancement of decolorization in non-sterile chip flasks may reflect synergistic effects of the combination of organisms. Previous studies of decaying wood debris conclude that basidiomycete fungi, bacteria, and yeasts in combination decay substantially more wood than any of these classes of organisms in isolation. Furthermore, the presence of bacteria and yeasts can have dramatic stimulatory effects on fungal growth (Blanchette and Shaw 1978a and 1978b). Field applications of fungi to treat PME will necessarily be carried out in non-sterile field conditions. While the results of the non-sterile treatment are clouded somewhat by the problematic sterile chip flask results, there is an indication that *Acremonium sp.*, rather than being neutralized or eliminated in non-sterile conditions, may be able to enhance decolorization of PME when in contact with organisms present in the normal waste stream. Chip assay contrast analysis implies further that

non-sterile conditions may in general be capable of providing higher decolorization of PME than sterile conditions.

Results of this study suggest that *Acremonium sp.* has potential for a cost-effective biological treatment of the dark color of pulp mill effluent. Decolorization by 44% is a dramatic, visible change from dark brown to golden yellow, and the fungus was able to effect this change at room temperature, without aeration or pH adjustment. However, further study is needed to bring about a field application. Vigorous establishment of the fungus as a biofilm on stationary medium will be required for any useful application, and it remains to be explored what type of medium will be effective. Bench-scale applications of *Phanerochaete chrysosporium* generally establish thick fungal mats on RBC plates or polyurethane foam cubes by inoculation with spore suspension before exposure to effluent (e.g., Joyce et al. 1984, Messner et al. 1990). The difficulty of establishing *Acremonium sp.* on Douglas-fir chips in this study may indicate a necessity to establish dense colonization under sterile conditions before exposing them to other organisms. Or, an alternative medium might be more effective.

Nutrient requirements are disadvantageous for any biological treatment. In this study, *Acremonium sp.* was unable to show dramatic decolorization without the addition of glucose. Investigation of cheaper nutrient sources will be essential if a practical application is to be devised.

The analysis of decolorization rate carried out in Run 3 indicates that a five-day residence will be the most efficient exposure time. This might be decreased by higher ratios of fungal surface area to effluent volume than used in this study. Examination of the biochemistry and kinetics of decolorization by *Acremonium sp.*, and an understanding of its metabolic processes will be necessary to maximize the efficiency of the retention time

used. While this work was carried out with constructed wetlands in mind, the significant enhancement due to aeration suggests that a fungal application might be most efficient if placed in an aerated secondary treatment lagoon. Alternatively, a constructed wetland application might include passive aeration such as waterfalls.

Seasonal variations in PME may also affect fungal treatability. This may indicate an advantage in using fungi only during the summer, when the effectiveness of fungal enzymes is highest and, simultaneously, wastewater discharge permits are most strict.

Finally, a striking corollary suggestion of this work is the possibility of other fungal species with potential for treatment of this wastewater. Hartig and Lorbeer (1991) have listed pages of lignin-degrading fungi; most of them have not been tested for PME treatment capability. Work with *P. chrysosporium* certainly broke ground in the arena of biological treatment, and it continues to show promise for applications such as biopulping, biobleaching, and hazardous waste cleanup. But perhaps it is now time to acknowledge some of the limitations of this organism and seek others which are better suited to the wastewater environment. *Acremonium sp.* might represent one of a multitude of other organisms which could cost-effectively provide pulp mill effluent decolorization.

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APPENDICES

APPENDIX A: Conversion table

5 g/l glucose* = 0.5% = 27.8 mM glucose = 167 mM C
10 g/l glucose = 1% = 55.6 mM glucose = 334 mM C
20 g/l glucose = 2% = 111.2 mM glucose = 668 mM C

50.4 mg/l urea = 0.00504% = 0.839 mM urea = 1.68 mM N
100 mg/l urea = 0.01% = 1.67 mM urea = 3.33 mM N
200 mg/l urea = 0.02% = 3.34 mM urea = 6.68 mM N

*Fructose conversions are exactly the same as glucose conversions

APPENDIX B: Data and statistical analysis
(All data reported in platinum-cobalt color units, PCU)

December 1991 - Run 1

Initial effluent color 3435

DATA

Color after 14 day decolorization period

Four replicates per treatment (five control)

Fungus:		Nutrient level: a	b	c	d	e
	Angelou	3121	2945	2933	3221	3242
		3129	3037	2979	3213	3271
		3346	3087	2787	3275	3263
		3179	2887	2954	3296	3304
	Acremonium sp.	3150	3000	3029	3204	3238
		3192	3046	3004	3238	3196
		3200	3012	3004	3217	3167
		3263	3029	3058	3242	3154
	Phanerochaete	3263	2895	3188	3229	3259
	chrysosporium	3217	2954	3204	3238	3246
	BKM F-1767	3225	3087	2895	3179	3250
		3213	2954	2983	3137	3238
	Phlebia	3200	2958	3025	3175	3192
	subserialis	3217	3054	3263	3087	3204
	SS3	3188	3526	3171	3179	3254
		3234	3380	3208	3096	3204
	Control	3117	3238	3279	3250	3238

Statistical Analysis - Run 1 Data

ANOVA

Source	d.f.	SS	MS	F
Total	84	1385614		
Treatment	20	913582	45679.1	6.19**
Residual	64	472032	7375.5	

r. = 85 $C = y_{..}^2/r. = 8.44 \times 10^8$

Compared ranked means with Bayes LSD:

q=20 f=64≈60 F=6.19≈6.0 k=500 (0.01 significance level)

minimum average risk t_B=2.56[§]

BLSD = 155

Control mean = 3224

All treatment means below 3224-155=3069 are significant @ 0.01 level

TREATMENTS SIGNIFICANTLY (0.01) DIFFERENT FROM CONTROLS:

Angelou, Nutrient levels b & c

Acremonium sp., Nutrient levels b & c

P. chrysosporium, Nutrient levels b & c

**Significant @ 0.01 level

[§]Petersen, 1985, p. 415.

March 1992 - Run 2

Initial effluent color: 2926

DATA

Color after 14 day decolorization period

Four replicates per treatment

Fungus:		Nutrient level: f	g	h	e
	Angelou	2712	2778	2874	2920
		2904	2678	2837	3054
		2758	2962	2795	2920
		2720	2887	2841	2900
	Acremonium sp.	2941	1731	2858	2812
		2883	1735	2887	2837
		2829	1664	3008	2841
		2691	1672	3004	2824
	Phanerochaete	2983	2778	2912	2945
	chrysosporium	2766	2833	2762	2879
	BKM F-1767	2854	2983	2804	2900
		2745	2833	2887	2900
	Phlebia	3004	3096	2783	2958
	subserialis	3016	3012	2824	2870
	SS3	2849	2891	2808	2874
		2925	2778	2887	2925
	Control	2762	2845	2733	2824

Statistical Analysis - Run 2 Data

ANOVA

Source	d.f.	SS	MS	F
Total	67	5628011		
Treatment	16	5311106	331944	54.1**
Residual	51	316904	6214	

r. = 68 $C = y..^2/r. = 5.32 \times 10^8$

Comparing ranked means with Bayes LSD:

q=16≈20 f=50≈40 F=54.1≈25 k=500 (0.01 significance level)

minimum average risk $t_B = 2.41$[§]

BLSD = 134

Control mean = 2791

All treatment means below 2791-134=2657 are significant @ 0.01 level

TREATMENTS SIGNIFICANTLY (0.01) DIFFERENT FROM CONTROLS:

Acremonium sp., Nutrient level g

**Significant @ 0.01 level

[§]Petersen, 1985, p. 415.

June 1992 data - Run 3

Initial effluent color: 3388

DATA

Color after 14 day decolorization period

Four replicates per treatment (five control)

Fungus:		Nutrient level: i	g	g	j	k
	Angelou	2628	2787	3321	3041	2344
		2549	2632	2724	2119	2687
		2319	3062	3413	1685	2657
		2624	3213	2770	2453	2791
	Acremonium sp.	1964	2674	1839	1919	1881
		1914	2624	2265	1864	1994
		1935	2031	2282	1894	1877
		1881	2311	2052	1864	2006
	Phanerochaete	2282	2507	1860	1960	2620
	chrysosporium	2687	2916	2699	2365	2774
	BKM F-1767	2570	2703	2695	2490	2845
		2186	2582	2432	1873	2883
	Control	2749	3071	2837	2954	2895

Statistical Analysis - Run 3 Data

ANOVA

Source	d.f.	SS	MS	F
Total	64	11721741		
Treatment	15	8447451	563163	8.43**
Residual	49	3274290	66822	

r. = 65 $C = y_{..}^2/r. = 3.91 \times 10^8$

Comparing ranked means with Bayes LSD:

q=15≈20 f=49≈40 F=8.43≈10.0 k=500 (0.01 significance level)

minimum average risk $t_B = 2.5$§

BLSD = 457

Control mean = 2901

All treatment means below 2901-457=2444 are significant @ 0.01 level

TREATMENTS SIGNIFICANTLY (0.01) DIFFERENT FROM CONTROLS:

Angelou, Nutrient level j

Acremonium sp., Nutrient levels g1, g2, i, j, & k

P. chrysosporium, Nutrient levels g2 & j

**Significant @ 0.01 level

§Petersen, 1985, p. 415.

Dec 1992 - Run 4

Initial effluent color: Sterile 4185
Non-ster 4448

DATA

Color after 14 day decolorization period
Five replicates per treatment (three control)

		NON-AERATED			
		STERILE		NON-STERILE	
		Nutrient level:		Nutrient level:	
		g	j	g	j
Fungus:					
	Acremonium sp.	3734	3638	3759	3764
		2933	4064	4043	3655
		3446	3330	4006	3972
		3075	2870	4014	3697
		2912	3000	3847	3797
	Phanerochaete		4336		3839
	chrysosporium		3709		3647
	BKM F-1767		4319		3814
			4198		3689
			4327		3689
	Control		4256		3989
			4248		3693
			4319		3864

		AERATED			
		STERILE		NON-STERILE	
		Nutrient level:		Nutrient level:	
		g	j	g	j
Fungus:					
	Acremonium sp.	2912	2269	3726	3672
		2703	2290	3739	3534
		2165	2131	3613	3655
		2098	2303	3467	3638
		2106	2286	3647	3442
	Phanerochaete		4056		3684
	chrysosporium		3968		3651
	BKM F-1767		3972		3709
			3985		3663
			2996		3676
	Control		4177		3785
			4043		3722
			3931		3780

Statistical Analysis - Run 4 Data

ANOVA

Source	d.f.	SS	MS	F
Total	71	25475037		
Treatment	15	22001919	1466795	23.65**
Residual	56	3473119	62020	

r. = 72 $C = y_{..}^2/r. = 9.08 \times 10^8$

Comparing ranked means with Bayes LSD:
q=15≈20 f=56≈60 F=23.65≈25 k=500 (0.01 significance level)

minimum average risk t_B=2.36§
BLSD = 372

Control means:
Sterile, non-aerated = 4274 Non-st., non-aerated = 3849
Sterile, aerated = 4050 Non-st., aerated = 3762

All treatment means below (Control-BLSD) are significant @ 0.01 level

TREATMENTS SIGNIFICANTLY (0.01) DIFFERENT FROM CONTROLS:
Acremonium sp., Sterile, aerated and non-aerated,
Nutrient levels g & j

**Significant @ 0.01 level
§Petersen, 1985, p. 415.

Analysis of selected data
Run 3, Acremonium sp. and P. chrysosporium AND Run 4, all sterile

Color reported as fraction of control

(Control - Data value)
Control

Fungus:	Run 3 Nutrient level: g1	g2	i	j	k	Run 4 Nutrient level: g	j	g (Aer)	j (Aer)
Acremonium sp.	0.0778	0.3637	0.3208	0.3366	0.3494	0.1258	0.1481	0.2798	0.4378
	0.0949	0.2179	0.338	0.3552	0.3108	0.3125	0.0489	0.3311	0.4326
	0.298	0.2122	0.3309	0.3452	0.3509	0.1929	0.2201	0.4634	0.4716
	0.2022	0.2908	0.3494	0.3552	0.3065	0.2794	0.3271	0.4798	0.4295
						0.3173	0.2969	0.4778	0.4337
Phanerochaete	0.135	0.3566	0.2122	0.3223	0.0964		-0.0143		-0.0014
chrysosporium	-0.005	0.0692	0.0735	0.1836	0.0435		0.1316		0.0202
BKM F-1767	0.0678	0.0706	0.1135	0.1407	0.0192		-0.0104		0.0192
	0.1092	0.1607	0.2451	0.3523	0.0063		0.0178		0.0161
							-0.0123		0.2592

Contrasts tested on subset of selected data
[Run 3, all *Acremonium sp.* and *P. chrysosporium*; and Run 4, all sterile.]

1.	BgA Run 3 vs. 4	Variability between Runs
2.	BjA Run 3 vs. 4	
3.	CjA Run 3 vs. 4	
4.	A vs. A	Overall effect of aerating
5.	BA/CA vs. BA/CA	Interaction of aeration/fungus (678 2X2)
6.	B vs. C	Overall difference between B & C fungi
7.	gj vs. ik	Difference between a mid-level of glu (10) and high/low (5/20) levels
8.	Bgj/Cik vs. Bik/Cgj	Interaction of nutrient level/fungus (8910 2X2)
9.	g vs. j	Effect of changing urea level when glu=10 mg/l
10.	i vs. k	Difference between high and low glu levels

ANOVA Table: Analysis of Contrasts of Selected Data

Source	d.f.	SS	MS	F
Total	69	1.5191		
Among treatments	15	1.1678	0.0779	11.97**
Contrasts: #1,2,3 (pooled)	3	0.1726	0.0575	8.84**
#4	1	0.1329	0.1329	20.4**
#5	1	0.0279	0.0279	4.29*
#6	1	0.6443	0.6443	99.0**
#7	1	0.0005	0.0005	0.077
#8	1	0.0796	0.0796	12.2**
#9	1	5.48×10^{-5}	5.48×10^{-5}	0.008
#10	1	0.0159	0.0159	2.44
Run 3: g1 vs. g2 results	2	0.0364	0.0182	2.80
Residual	54	0.3513	0.00651	

*Significant @ 0.05 level
**Significant @ 0.01 level

July 1992 - Chip Assay 1

Initial effluent color 3313

Fungus (Chip soak):	Color units
Acremonium sp.	3137
(1.5% malt extract)	3008
	2916
Acremonium sp.	3033
(1% glucose,	2983
0.01% urea)	2887
Control	3008
(dH2O)	2979
	2841

Statistical Analysis - Chip Assay 1 Data

ANOVA

Source	d.f.	SS	MS	F
Total	8	61135		
Treatment	2	9238	4619	0.534
Residual	6	51897	8650	

r. = 9 C = 7.98×10^7

No significant treatment effect.

August 1992 - Chip Assay 2

STERILE CHIP FLASKS

Initial effluent color 3409

Five replicates per treatment (eight control)

Fungus:	Incubation time (days) 2	4	9	12
Acremonium sp.	2883	3092	3279	2958
	3075	3279	2703	2657
	3296	3008	2908	3342
	2858	3292	2795	2816
	3058	2812	3133	2662
Phanerochaete	2900	3029	2841	
chrysosporium	3050	3008	2912	
BKM F-1767	3083	3025	3079	
	3012	2925	2941	
	2941	3004	3008	
Control	2937	3196	3071	3250
	4139	3179	3709	3296

NON-STERILE CHIP FLASKS

Initial effluent color 3246

Seven replicates per treatment

Acremonium sp.	2365
(9-d incubation)	2315
	2236
	2386
	2365
	2286
	2361
Control	2478
	2607
	2766
	2595
	2503
	2687
	2545

Statistical Analysis - Chip Assay 2 Data & Selected Contrasts

ANOVA

Source	d.f.	SS	MS	F
Total	56	6.963×10^6		
Treatment	9	4.920×10^6	5.466×10^5	12.57**
Among *Acremonium sp.*	3	122403	40801	0.940
Among *P. chrysosporium*	2	5660	2830	0.065
Acr. sp. vs. *P. chry.*	1	1130	1130	0.026
Between Non-sterile	1	2.487×10^5	2.487×10^5	5.73*
St. Treatment vs. Control	1	8.291×10^5	8.291×10^5	19.10**
Non-sterile vs. St.	1	3.424×10^6	3.424×10^6	78.89**
Residual	47	2.044×10^6	4.348×10^4	

$r. = 57$ $C = y..^2/r. = 4.831 \times 10^8$

Comparing ranked treatment means with Bayes LSD:
$q=9 \approx 6$ $f=47 \approx 40$ $F=12.57 \approx 10$ $k=100$ (0.05 significance level)
→ minimum average risk $t_B=1.9$§

BLSD = 251

Control means: Sterile = 3347 Non-sterile = 2346
All treatment means below (Control-BLSD) are significant @ 0.05 level

TREATMENTS SIGNIF. (0.05) DIFFERENT FROM MATCHED CONTROLS:
Acremonium sp., Sterile: 2, 9, & 12 day incubation, and Non-sterile
P. chrysosporium, Sterile: 2, 4, & 9 day incubation

Comparing selected contrasts per Steel and Torrie (1960)

SIGNIFICANT CONTRASTS:
Non-sterile *Acremonium sp.* vs. non-sterile control*
Sterile fungal treatments vs. sterile controls**
Non-sterile treatments and controls vs. sterile treatments and controls**

*Significant @ 0.05 level
**Significant @ 0.01 level
§Petersen, 1985, p. 413